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Cara Marks

PI - Signature Date

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Summary

Breast cancer occurs when there is a transition from normal breast epithelial cell behavior to that of uncontrolled cell growth. Cell surface receptors and specific growth factors play a crucial role in this transition. In healthy tissue, cell surface receptors transmit a growth signal upon growth factor or ligand binding. Ligand binding promotes contact between adjacent receptors leading to receptor dimerization. Extracellular receptor dimerization stabilizes interactions between cytoplasmic domains and leads to activation of the receptors intracellular protein tyrosine kinase. Overexpression of growth factor receptors can lead to spontaneous oligomerization, triggering of the protein kinase cascade, and the unregulated growth characteristic of cancer. Indeed the most common cellular lesion found in human cancers involves autocrine activation in conjunction with receptor overexpression. Overexpression of growth factor receptors has been reported on breast, prostate, ovarian, bladder, pancreatic and lung cancers. The epidermal growth factor (EGF) family of receptors (EGFR or ErbB, ErbB2, ErbB3, and ErbB4) has been implicated more than any other in the development of human carcinomas. Signal transduction can occur from either homo- or heterodimerization of members of the EGF family of receptors. In approximately 30% of human breast cancers, the EGFR receptor is overproduced, resulting in aggressive uncontrolled growth of breast tumor cells. Whether this is due to homo- or heterodimerization is unclear. Regardless, EGF receptors play a crucial role in the transition from normal cell behavior to that of uncontrolled cell growth.

Our research addresses the pathogenesis of breast cancer, through an examination of the structural basis of EGFR dimerization leading to abnormal rapid growth characteristic of cancer. This analysis will not only increase our understanding of the mechanism by which EGFR overexpression results in unregulated breast cell proliferation, but may also provide insights on the mechanism of action of other growth factor receptors. Knowledge of the atomic structure of the EGFR dimer interface will also permit the rationale design of small molecule EGFR oligomerization antagonists capable of arresting breast cancer growth.

Solving the structure of EGFR also has tremendous translational potential for development of breast cancer therapy. An anti-ErbB2 monoclonal antibody developed by Genentech Inc. has been approved by the FDA for breast cancer therapy. This monoclonal antibody exerts its anti-tumor effect by removing ErbB2 from the cell surface. Knowledge of the atomic structure of the EGFR dimer interface would permit the rationale design of small molecule EGFR dimerization antagonists capable of arresting breast cancer growth. A small molecule EGFR antagonist would have distinct pharmacokinetic, bioavailability, economic, and tumor penetration advantages compared to a monoclonal antibody.

Background

Role of ErbB family of receptors in malignant transformation

The epidermal growth factor (EGF) family consists of four (ErbB, ErbB2, ErbB3, and ErbB4) 185 kDa protein tyrosine receptors whose prototype is the EGF receptor (ErbB). These receptors consist of a 650 amino acid extracellular domain (ECD) with two characteristic cysteine-rich regions, a single transmembrane domain and an intracellular kinase domain. Members of this family have been implicated more than other growth factor receptors in the development of human adenocarcinomas. Increased expression of the EGF receptor (ErbB) and ErbB2 have been associated with aggressive tumors of the stomach, bladder, lung and breast. 20% of breast cancers are malignant by virtue of their abnormally high expression of EGFR and overexpression correlates with aggressive cancer and poor prognosis [Rachwal, 1995 #189; Marcias, 1987 #237; Seshadri, 1996 #227]. Overexpression of either EGFR or ErbB2 correlates with poor prognosis [Rachwal, 1995 #189; Marcias, 1987 #237; Seshadri, 1996 #227; Slamon, 1987 #8; Slamon, 1989 #76]. Moreover, data from numerous studies suggest that EGFR family members may participate in a complex yet flexible network of signal transduction by forming heterodimers between family members [Earp, 1995 #225; Spivak-Kroizman, 1992 #238; Alimandi, 1995 #232]. These heterodimers may form even when only one receptor binds its ligand. Alternatively, cooperative signaling may explain why a specific ligand has not been identified which activates ErbB2. Cooperative signaling appears to play an important role in neoplastic transformation.

A. Methods

A.1. Expression and purification of EGFR ECD for crystallization and isolation of anti-EGFR antibodies T

Overexpress and purify EGFR

The 630 amino acid ECD of EGFR was cloned from a phagemid (American Type Culture Collection, Rockville, MD.) into the mammalian expression vector used to express and crystallize ErbB2 [McCartney, 1995 #147]. The vector contains the SV40 early promoter and a dihydrofolate reductase (DHFR) selectable amplifiable gene. The vector was transfected into Chinese hamster ovary (CHO) DHFR- cells. To facilitate the purification of expressed ECD protein by immobilized metal affinity chromatography (IMAC), the gene for EGFR ECD was placed upstream to a hexahistidine DNA sequence in the expression vector. The cell culture, transfection and amplification procedures followed the procedures descibed in [Hudziak, 1991 #233]. Colonies were selected and assayed for ECD expression by ELISA and those exhibiting the highest expression levels werefurther amlpified by methotrexate selection. Clones exhibiting the highest levels of expression were chosen for production of EGFR ECD Production medium was harvested every 3-5 days [McCartney, 1995 #147]. The EGFR ECD fused to the hexahistidine

peptide was purified by IMAC as described below. Aggregates will ere removed by FPLC size exclusion.

A.2. Isolation and initial characterization of human anti-EGFR antibodies from phage display library

Anti-EGFR antibody fragments will be isolated using several strategies including selection on a non-immune human scFv phage antibody library [Marks, 1991 #13] ,on whole cells producing EGFR, immobilized and soluble EGFR ECD. A phage antibody library containing 7.0×10^9 members has been provided by our collaborator, Dr. James D. Marks. Antibodies have been isolated from this library against all 10 antigens tested to date, with between 6 and 14 different antibodies isolated per antigen. The affinities of the scFv range between 5.0×10^{-8} M to 5.0×10^{-9} M. Antibodies can be selected in approximately two weeks, typically express at high level in *E. coli*, and can be easily engineered to create bivalent antibody fragments. Data presented in the preliminary results section validate this approach for isolating antibody fragments which can mediate crystallization. Anti-EGFR antibodies will be isolated by selecting the phage antibody library on EGFR-ECD immobilized on polystyrene as previously described [Schier, 1995 #122]. The phage library will be subjected to 4 rounds of selection, and 92 clones from each round of selection will be screened for binding to EGFR-ECD by ELISA as previously described [Schier, 1995 #122] Clones showing EGFR-ECD specificity will be further characterized by BstN1 fingerprinting [Clackson, 1991 #54] to determine the number of unique scFv and DNA sequencing.

5. Technical Objectives

Purification of EGFR ECD

- By SDS PAGE, major contaminanproteins following IMAC included:
  - ~200 K Da protein (possible aggregate)
  - ~120 K Da protein
  - major band at 60 K Da corresponding to BSA (cells grown in 4% FCS)
  - ~ 30 K Da protein

- 120 K Da protein was a weak band, BSA was the major contaminant, others could be separated by size exclusion chromatography in theory.

- Cells stopped producing EGFR upon lowering serum concentration to 3%FCS

- Ion exchange a popular method for purifying proteins, so we started there

- Literature/advice recommended starting at least 1 pH unit below the pI, then going to 1 pH unit above the pI: pI of EGFR ECD = 6.5
  pI of BSA = 5.3

Chromatography Experiments

SP Sepharose Fast Flow (Pharmacia) was chosen as a first step because it is a strong cation exchanger.. A 10 ml column was packed according to the

manufacturer's specifications on a Biocad FPLC. 10 mls of cell culture harvest containing the secreted form of the external domain of the EGF receptor was dialyzed overnight in 4 liters of 50 millimolar sodium acetate pH 5.0 to facilitate protein binding to the column. The dialyzed material was applied to the 10 ml SP Sepharose Fast Flow ion exchange column (Pharmacia) equilibrated in 50 mM sodium acetate pH 5.0 at a flow rate of 1 ml per minute. Proteins were eluted from the column in a sodium chloride gradient starting at 0 and ending at 1M salt over 60 minutes; fractions were collected in 1 ml volumes. The chromatogram revealed two major overlapping protein peaks, both broad and rounded. Fractions were analyzed for the presence of EGFR ECD by slot blot with anti-EGFR ECD #28 (Sugen). EGFR ECD-positive fractions roughly corresponding to the first peak were further analyzed by SDS-PAGE, revealing the presence of both high and low molecular weight contaminants in all fractions. The experiment was repeated several times to explore the effects of different buffers over a wide pH range. In each case, fresh cell culture harvest was dialyzed into the column starting buffer to maintain consistency. The fractions collected in each experiment were analyzed as previously described, first by slot blot and then by SDS-PAGE. In the case of 50 millimolar sodium acetate pH 5.5 there were two overlapping peaks with results similar to those obtained at pH 5.0. With 50 millimolar sodium phosphate pH 6.0, one broad peak was obtained that spanned twenty fractions. No separation of EGFR ECD from contaminants was observed by SDS-PAGE, probably because pH 6.0 is extremely close to the pI of EGFR ECD. Experiments run with 50 millimolar tris at both pH 7.0 and pH 8.0 yielded two broad overlapping peaks which contained EGFR ECD and contaminants. Overall, the strong cation exchanger SP Sepharose proved ineffective at separating major contaminants from the protein of interest.

POROS PI/M

Since the strong cation exchanger was unproductive over a wide range of pH, we decided to investigate the effect of a weak anion exchanger. 10 mls of cell culture harvest containing the secreted form of the external domain of the EGF receptor was dialyzed overnight in 4 liters of 50 millimolar tris pH 7.5 to facilitate protein binding to the column. The dialyzed material was loaded onto a commercially packed PI/M column (POROS) equilibrated in 50 millimolar tris pH 7.5 at a flow rate of 4 mls per minute. Proteins were eluted from the column in a sodium chloride gradient starting at 0 and ending at 1M salt over 60 minutes; fractions were collected in 2 ml volumes. The chromatogram revealed four major peaks, with the last three overlapping. Fractions were analyzed for the presence of EGFR ECD by slot blot with anti-EGFR ECD #28 (Sugen). The first two peaks contained EGFR ECD-positive fractions, which were further analyzed by

SDS-PAGE. The gel showed that the first peak eluted a low molecular weight contaminant (~66KDa) corresponding to bovine serum albumin, and the second peak eluted several high molecular weight contaminants (150 - 200 KDa) in addition to the EGFR ECD. The experiment was repeated at pH 8.0 and pH 8.5 to explore the effect of higher pH. In each case, fresh cell culture harvest was dialyzed into the column starting buffer to maintain consistency, The fractions collected in each experiment were analyzed as previously described, first by slot blot and then by SDS-PAGE. Similar results were obtained at each of the higher pHs. Although it yielded greater resolution and a larger number of peaks than the SP Sepharose, the POROS PI/M column proved ineffective at isolating EGFR ECD.

Q Sepharose (Pharmacia)

- Encouraged by the positive results with PI/M, we moved on to a strong anion exchanger.

- Packed a 10 ml column on FPLC similar to SP description

- Same strategy, attempted: pH 7.5 Tris, pH 8.0 Tris, pH 8.5 Tris

- Results: Two overlapping peaks, similar to SP Sepharose chromatograms, pH has no appreciable effect. Slot blot to determine which fractions contain EGFR ECD; SDS PAGE of relevant fractions shows BSA still contaminates protein of interest.

- Conclusion: BSA - Can't separate with ion exchange, must have similar charge, or might be sticking to EGFR ECD. Attempt affinity chromatography in hopes of removing BSA.

CM Blue Affinity Gel (Biorad)

- Bi-functional affinity/ion exchange matrix:
  Contains: 1. Cibacron blue – binds proteins with a dinucleotide fold, 1 2. CM (carboxymethyl) – cation exchanger removes serum proteases.

- Manufacturer's stated capacity = 4 mgs BSA/ 1 ml gel, no dialysis necessary

- Used as a first step to try and remove BSA

- Protocol: Pack 5 ml column and wash with 40% isopropanol to remove excess blue dye. Equilibrate in running buffer (10 millimolar potassium phosphate, 150 millimolar sodium chloride pH 7.25) Load 10 mls EGFR ECD cell culture harvest and collect flow-through. Wash 3X 5mls running buffer, collect separate fractions. Elute junk (BSA and serum proteases) with 10 mls 1.4Molar sodium chloride. Regenerate column with 10 mls 2Molar guanidine H Cl . Protein of interest should be in flow through, and BSA should have stuck. Analysis by SDS PAGE and western blot using anti-EGFR ECD #28 (Sugen). Gel shows that some of the BSA is removed by blue gel, but not all. There is still BSA in all fractions, including flow-through. Western reveals EGFR ECD in FT, wash #1 and a little bit in wash #2. Conclusion: Perhaps more gel is needed to remove all the BSA. Vary ratio of gel/harvest. Repeat experiment using 10 ml column, then 15 ml column. Same results. Conclusion: blue gel is effective at removing some BSA. Maybe this is enough.

- Try using blue gel as a first step, then the following second steps (using pooled FT and wash #1 dialyzed into starting buffer in each case):

....

- Conclusion: blue gel does not remove enough BSA to help.

Concavalin A Agarose (Vector Labs)

- Carbohydrate binding column, should bind EGFR ECD and let junk flow through

- Manufacturer's stated capacity = about 6 mgs protein/1ml gel

- Protocol: 10 mls of cell culture harvest was bound to 1 ml conA agarose equilibrated in TBS (50 mM Tris 7.5, 150 mM KCl) for one hour. Column was washed 3X 1ml TBS, then protein was eluted in 2.5 mls 0.5M alpha methyl manopyranoside. Samples were analyzed by Western: FT, washes and elution contained significant amounts of EGFR By SDS PAGE elution has some high and low mw contaminants (better than before). Try using conA as a first step, then dialyzing elution into starting buffer for:
    - Poros CM in MES pH 5.7
    - SP Sepharose in pH 8.0 Tris
    - Poros PI/M in pH 8.5 Tris
    - WGA (Wheat germ agglutin) binds different types of carbohydrates

RESULTS in all of the above cases, still contaminants.

- Repeat conA experiment under different conditions; 50 mls cell culture harvest bound overnight to 4 ml conA column equilibrated with 20 mM HEPES 7.5, 2% glycerol, then poured onto a disposable column. Wash 2 X 4mls buffer, then elute in 5 X 4ml fractions (0.5M alpha methyl manopyranoside). Samples again analyzed by Western and SDS PAGE. Results: more than 90% of the EGFR binds to the column, EGFR elutes as a 97 Kda protein with major peak in two column volumes. Yeah!!!! No contaminants.

- Conclusion: elution in TBS yielded contaminants, but changing the running buffer to a low ionic strength, using larger gel/harvest ratio, and binding overnight increases purity to 98% or more in a one step purification.

In summary, using this strategy, 8mgs of EGFR ECD was purified and is sufficient for intial crystallization trials and antibody selections. These series of experiments as underway.